

Fatal familial insomnia: mitochondrial and protein synthesis machinery decline in the mediodorsal thalamus

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Abstract

The expression of subunits of mitochondrial respiratory complexes and components of the protein synthesis machinery from the nucleolus to the ribosome was analyzed in the mediodorsal thalamus in seven cases of Fatal Familial Insomnia (FFI) compared with age-matched controls. NDUFB8 (complex I subunit), SDHB (complex II subunit), UQCRC2 (complex III subunit), COX2 (complex IV subunit) and ATP50 (complex V subunit) expression levels, as revealed by western blotting, were reduced in FFI. Voltage-dependent anion channel (VDAC) and ATP5H were also reduced due to the marked depopulation of neurons. In contrast, a marked increase in superoxide dismutase 2 (SOD2) was found in reactive astrocytes thus suggesting that astrocytes are key factors in oxidative stress responses. The histone-binding chaperones nucleolin and nucleoplasmin 3, and histone H3 di-methylated K9 were markedly reduced together with a decrease in the expression of protein transcription elongation factor eEF1A. These findings show severe impairment in the expression of crucial components of mitochondrial function and protein synthesis in parallel with neuron loss in mediodorsal thalamus at terminal stages of FFI. Therapeutic measures must be taken long before the appearance of clinical symptoms to prevent the devastating effects of FFI.

Key words: Fatal familial insomnia, mitochondria, protein synthesis, mitochondrial respiratory chain, nucleolus, ribosome

Introduction

Prion protein is encoded by *PRNP* located on the short arm of chromosome 20 in humans. The second exon of *PRNP* encodes a protein of 253 amino acids that is truncated post-translationally to remove twenty-two amino acids at the amino-terminal and twenty-three amino acids at the carboxyl-terminal, being replaced at this site by the addition of a glycosylphosphatidylinositol (GPI) anchor. Additional putative glycosylation in two asparagine sites gives rise to three isoforms, fully di-glycosylated, mono-glycosylate and non-glycosylated prion protein (3). Mutations in *PRNP* are causative of genetic prion diseases, some of which are manifested as familial Creutzfeldt-Jakob disease (CJD), the phenotype of which depends on the site of *PRNP* mutation and also on the polymorphism of codon 129 in *PRNP* (3).

Fatal familial insomnia (FFI) is an autosomal dominant prion disease caused by a D178N mutation in *PRNP* in combination with methionine (Met) at codon 129 in the mutated allele of the same gene (D178N-129M haplotype) (15, 23, 24, 26, 27, 31). Homozygosity, either methionine-methionine or valine-valine, at codon 129 in the normal allele of *PRNP* results in acceleration of clinical symptoms and shorter duration of the disease (5, 13, 29).

Clinically, FFI is principally manifested as sleep disturbances with insomnia, sleep fragmentation, and altered arousal and dreaming, accompanied by autonomic disturbances including increased salivation and sweating, tachycardia, hypertension and impotence, as well as spontaneous and evoked myoclonus, among other neurological symptoms (5, 12, 14, 17, 20, 21, 29, 38).

Clinical symptoms reflect major atrophy of the anterior ventral and mediodorsal limbic nuclei of the thalamus that extends to the pulvinar, ventral anterior and ventral medial thalamic nuclei with disease progression. The inferior olives are the other major targets in FFI. Severe neuron loss and astrogliosis without spongiform change in these regions, in the context of familial disease, are the neuropathological characteristics of FFI. Moderate astrogliosis without neuron loss is common in the periaqueductal gray matter and hypothalamus. Involvement of the inner temporal cortex, including CA1 region of the hippocampus, cingulate cortex and other areas of the neocortex, is variable and depends on the duration of the disease, which in turn largely depends on the codon 129 polymorphism (14, 21, 25, 27, 29).

The deposition of abnormal prion protein (PrP^{Sc}) varies from one region to another in FFI. Immunohistochemistry usually shows small granular deposits in the temporal neocortex in cases with relatively long duration, whereas PrP^{Sc} deposition in thalamus is very scanty, if present. However, western blotting identifies a particular pattern

characterized by a weak band of proteinase-resistant non-glycosylated PrP^{Sc} at 19 kDa (type 2 PrP) and relatively strong bands of mono-glycosylated and di-glycosylated PrP^{Sc} which disappear following PNGase digestion, giving rise to a robust non-glycosylated band (14, 29, 30, 31).

Recent studies of gene expression profiling have shown altered gene expression in the thalamus in FFI cases when compared with controls. The main changes correspond to the following biological processes: transcription, regulation of transcription, protein biosynthesis, protein folding, protein transport, RNA splicing, electron transport chain, oxidative phosphorylation, energy metabolism, transport, and oxidation reduction (37). Proteomics methods have also been employed to assess altered protein profiles in FFI. Proteins involved in protein export and oxidative phosphorylation, as well as proteins involved in other neurodegenerative diseases as Alzheimer's disease and Parkinson's disease have been identified in the cortex and cerebellum (35). Unfortunately, the thalamus was not examined.

Because of the limited knowledge about altered metabolic pathways in FFI, the present study was designed to learn about alterations of two major complex pathways, one regulating energy metabolism, and particularly mitochondrial function, and the other protein synthesis. The study was performed on post-mortem samples of the thalamus of seven well-characterized cases of FFI which were analysed using combined qRT-PCR, western blotting and immunohistochemistry.

Material and methods

Human samples

All the cases were from the Basque Country in the north of Spain where FFI has a relative high incidence due to a founder effect in a historically small rural community with not uncommon endogamy (32). This study was focused on the mediodorsal thalamus from seven FFI cases and seven controls (Table I).

All FFI cases were males, had the mutation D178N and were Met/Met homozygous at the codon 129 of *PRNP*. The most common age at onset was between 47 and 57 years (five cases) with the exception of one young person aged 36 and one old individual aged 70. The duration of the disease was between 6 and 16 months independently of the age at onset.

Brain tissue was obtained from the Brain Bank of the Araba University Hospital and Basque Biobank for Research (O+eHun) and the Institute of Neuropathology Biobank following the guidelines of the Spanish legislation on this matter and the approval of the local ethics committees. The post-mortem interval between death and tissue processing was between 4h 15min and 22h 50min. One hemisphere was immediately cut in coronal sections, 1cm thick, and selected areas of the encephalon were rapidly dissected, frozen on metal plates over dry ice, placed in individual air-tight plastic bags, numbered with water-resistant ink and stored at -80°C until use. The other hemisphere was fixed by immersion in 4% buffered formalin for 3 weeks. Neuropathological examination in all cases was routinely performed on twenty selected de-waxed paraffin sections comprising different regions of the cerebral cortex, diencephalon, thalamus, brain stem and cerebellum which were stained with haematoxylin and eosin, Nissl staining, and for immunohistochemistry to microglia using antibodies Iba1 and CD68, glial fibrillary acidic protein (GFAP), β -amyloid, phosphorylated tau (clone AT8), PrP (using the 3F4 antibody without and with pre-treatment with proteinase K), α -synuclein, TDP-43, ubiquitin and p62. FFI cases were pre-treated with formic acid. Age-matched control cases had not suffered from neurologic, psychiatric, or metabolic diseases (including metabolic syndrome), and did not have abnormalities in the neuropathological examination excepting sporadic Alzheimer's disease-related pathology stages I-II/0 of Braak and Braak.

RNA extraction

RNA was obtained from about 100mg of the dorsomedial thalamus of FFI cases and controls using RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, GE) following the protocol provided by the supplier. All samples were treated with RNase-free DNase Set (Qiagen) for 15min to eliminate genomic DNA contamination. The concentration of

each sample was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) at 340 nm. RNA integrity was assessed with the RNA Integrity Number (RIN value) determined with the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) (34). RIN values were similar in control and FFI cases (Table I).

Retro-transcription reaction

Retro-transcription reaction of RNA samples was carried out with the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) using 1,000ng of RNA for each sample following the protocol provided by the supplier using a Gene Amp 9700 PCR System thermos-cycler (Applied Biosystems). RNA samples without reverse transcriptase were processed in parallel as controls of the reaction. No amplifications were obtained in any cases.

Real-time polymerase chain reaction (qRT-PCR)

Quantitative RT-PCR assays were performed in duplicate on cDNA samples obtained from the retro-transcription reaction diluted 1:20 in 384-well optical plates (Kisker Biotech, Steinfurt, GE) using the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). The reactions were carried out using 20xTaqMan Gene Expression Assays for genes involved in protein synthesis, energetic metabolism and purine metabolism, and 2xTaqMan Universal PCR Master Mix (Applied Biosystems). TaqMan probes used in the study are shown Table II. The reactions were conducted with the following parameters: 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 sec and 60°C for 1 min. The data were captured using the Sequence Detection Software (SDS version 2.2, Applied Biosystems). Threshold cycle (CT) data for each sample were analysed with the Livak Method or double delta CT ($\Delta\Delta CT$) method (19). First, delta CT (ΔCT) values were calculated as the normalized CT values for each target gene in relation to the endogenous control X-prolyl aminopeptidase P1 (*XPNPEP1*) used as house-keeping gene (9). Second, $\Delta\Delta CT$ values were obtained from the ΔCT of each sample minus the mean ΔCT of control samples. The fold change was determined using the equation $2^{-\Delta\Delta CT}$. Mean fold-change values for every region between FFI cases and controls were analysed with the Student's t test using GraphPad Prism 6 when the distribution was normal as revealed by the Kolmogorov-Smirnov test. If the distribution was not normal, mean fold-changes were analysed with the Mann-Whitney Test. Differences between groups were considered statistically significant at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Protein extraction and western-blotting

Thalamus samples (0.1g) were homogenized using lysis buffer composed of 100mM Tris pH 7, 100mM NaCl, 10mM EDTA, 0.5% NP-40 and 0.5% sodium deoxycolate plus protease and phosphatase inhibitors (Roche Molecular Systems, Pleasanton, CA,

USA). Samples were centrifuged at 4°C for 5 min at 10,000g USING Eppendorf centrifuge 5417R (Eppendorf®, Hamburg, GE) and the supernatants obtained were stored at -80°C. Proteins were separated in sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Control of protein loading was carried out with Coomassie blue staining. Proteins were then electrophoretically transferred to nitrocellulose membranes using the Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad) at 60mA/membrane for 90 min). Non-specific bindings were blocked by incubation in 5% milk in Tris-buffered saline (TBS) containing 0.2% Tween for 1 h at room temperature. After washing, the membranes were incubated at 4°C overnight with one of the primary antibodies listed in Table III in TBS containing 5% albumin and 0.2% Tween. Afterwards, the membranes were incubated for 1 h with the appropriate HRP-conjugated secondary antibody (1:1,000, Dako, Glostrup, Denmark), and the immune complexes were visualized with a chemiluminescence reagent (ECL, Amersham, GE Healthcare, Buckinghamshire, UK). Densitometry of western blot bands was assessed with the TotalLab program (TotalLab Quant, Newcastle, UK) and analysed with Graphpad Prism using Student's t test when the distribution was normal and Mann-Whitney test when the distribution was not normal, as assessed with the Kolmogorov-Smirnov normality test. Differences were considered statistically significant at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Immunohistochemistry

Immunohistochemical study was performed in 4 µm-thick dewaxed paraffin sections of the thalamus. Endogenous peroxidases were blocked with peroxidase (Dako, Glostrup, Denmark) followed by 10% normal goat serum. The primary antibodies are shown in Table III. Following incubation with the primary antibody at room temperature overnight, the sections were incubated with EnVision+ system peroxidase (Dako) at room temperature for 15min. The peroxidase reaction was visualized with diaminobenzidine (DAB) and H₂O₂. No antigenic peptides were available to carry out pre-adsorption studies of primary antibodies. However, the omission of the primary antibody in some sections was used as a control of the immunostaining; no signal was obtained after incubation with only the secondary antibody. Sections were slightly counterstained with haematoxylin. Quantification of Nissl-stained and immunoreactive cells identified with antibodies against glial fibrillary acidic protein (GFAP), CD68, voltage dependent anion channel (VDAC), nucleolin (NCL), nucleoplasmin 3 (NPM3), histone H3K9me2, ATP5H protein and superoxide dismutase 2 (SOD2) was carried out in the mediodorsal thalamus in five controls and five FFI cases (two fields per case) at a magnification of x200 which corresponds to an area of 450x280 µm². Results were expressed as mean values ± standard deviation (SD).

Results

1. General neuropathological findings

Severe neuronal loss and marked astrocytic gliosis was observed in every case in the mediodorsal and anterior nuclei of the thalamus, and inferior olives; the ventral nuclei of the thalamus and the entorhinal cortex were variably affected (five and six of seven cases, respectively). Spongiform change was not seen in the mediodorsal thalamus and only in the entorhinal cortex when affected. PrP. Discrete astrogliosis was also found in the periaqueductal grey matter. Representative changes in the mediodorsal thalamus and entorhinal cortex compared with corresponding regions in age-matched controls are shown in Figure 1. Neuron loss in the mediodorsal thalamus was severe accounting for about 80-90%; only isolated small neurons can be seen in some cases. Microglial response was analyzed using the antibodies Iba1 which identifies microglia and CD68 which recognizes active microglia. Increased numbers of Iba1- and CD68-immunoreactive cells was found in the mediodorsal thalamus, particularly in two of seven cases, and entorhinal cortex in the six cases in which this region was affected. Details of cell types are recognized at higher magnification (Fig. 1 inserts)

Immunohistochemistry using the 3F4 antibody disclosed synaptic and fine granular proteinase-resistant PrP immunoreactivity in the entorhinal cortex, whereas PrP immunoreactivity was absent in the mediodorsal thalamus in FFI cases (Fig. 2). As expected no proteinase-resistant PrP was present in control cases (data not shown).

2. Mitochondria and respiratory chain

2a. mRNA expression

Eleven genes which codify for proteins that are part of electron transport chain complexes, one gene that encodes a translocase of the mitochondrial outer membrane (*TOMM40*) and two genes that codify for lysosomal ATPase (*ATP6V0B* and *ATP6V1H1*) were analysed in the thalamus of FFI samples. Complex I was represented by *NDUFA2*, *NDUFA7*, *NDUFA10*, *NDUFB3*, *NDUFB7*, *NDUFB10*, *NDUFS7* and *NDUFS8*; complex II by *SDHB*; complex III by *UQCRC1* and *UQCRCB*; complex IV by *COX7A2L* and *COX7C*; and complex V by *ATP5D*, *ATP5G2*, *ATP5H*, *ATP5L* and *ATP5O*. Only *ATP5D* was down-regulated (Student's t test $p < 0.01$). Detailed data are shown in Supplementary Table I.

2b. Protein expression of selected mitochondrial proteins

2b1: Expression levels of mitochondrial proteins in mediodorsal thalamus.

NDUFB8 (complex I subunit), SDHB (complex II subunit), UQCRC2 (complex III

subunit), COX2 (complex IV subunit) and ATP50 (complex V) expression levels, as revealed by western blotting, were reduced in mediodorsal thalamus in FFI, whereas UQCRB (complex III) and ATP5A (complex V subunit) expression was similar in FFI and controls. Densitometric analysis using β -actin and VDAC for normalisation showed similar results. The only exception between β -actin and VDAC normalisation was ATP5O which presented significant differences when using β -actin between control group and FFI group but only a tendency with a p-value 0.09 when using VDAC for normalisation. Together, these results indicated that reduced protein expression of proteins of the respiratory chain was not merely the consequence of a reduced number of mitochondria, but was also related to the selective loss of certain vulnerable subunits (Figure 3). Detailed data are shown in Supplementary Table II.

2b2 Voltage-dependent anion channel (VDAC), ATP5H and superoxide dismutase 2 (SOD2) in mediodorsal thalamus in FFI

Consecutive sections were stained with Nissl staining and processed for immunohistochemistry to VDAC, ATP5H and SOD2. Nissl staining served to illustrate the severe loss of neurons in the mediodorsal thalamus in FFI when compared with controls, together with the increase in the number of small nuclei corresponding, in part to astrocytes (Fig. 4 A, B).

In control brains, neurons presented strong VDAC immunoreactivity, punctuate ATP5H immunoreactivity and weak SOD2 immunoreactivity in the cytoplasm. Marked decrease in VDAC immunoreactivity related to the loss of neurons was observed in the mediodorsal thalamus in FFI when compared with controls (Figure 4 C, D). This was accompanied by general decrease of ATP5H immunoreactivity due to neuron loss together with enhanced visualization of ATP5H immunostaining in reactive astrocytes (Figure 4 E, F). Marked SOD2 immunoreactivity was found in reactive astrocytes (Figure 4 G, H). Details of cell types are recognized at higher magnification (Fig. 4 inserts). Quantitative data are shown in Table IV.

3. Molecular pathways involved in protein synthesis

3a. mRNA expression of ribosomal proteins RPL and RPS, nucleolar proteins, and 18S rRNA and 28S rRNA

Nucleolin (*NCL*), nucleophosmin (*NPM1*) and eukaryotic upstream binding factor (*UBTF*) gene expression levels were analysed in FFI cases and controls. *NPM1* was up-regulated in the thalamus in FFI (Figure 5).

28S rRNA was also up-regulated in FFI cases (Figure 5).

The expression of nine RPL (*RPL5*, *RPL7*, *RPL21*, *RPL22*, *RPL23A*, *RPL26*, *RPL27*, *RPL30* and *RPL31*) and seven RPS (*RPS3A*, *RPS5*, *RPS6*, *RPS10*, *RPS13*, *RPS17* and *RPS20*) genes was assessed in the mediodorsal thalamus in FFI cases and controls. *RPS17* and *RPS20* were up-regulated (Figure 5). Detailed data are shown in Supplementary Table III.

3b. Immunohistochemistry of selected molecules involved in protein synthesis

In control mediodorsal thalamus, anti-NCL antibodies weakly decorated the nucleolus and heavily the cytoplasm of neurons. NCL immunoreactivity was weak if present in glial cells under the same staining conditions. Anti-nucleoplasmin 3 (NPM3) antibodies decorated the nucleolus of neurons. Histone H3 di-methylated K9 (H3K9me2), which modulates NCL and NPMs function in rRNA transcription (Tamada et al., 2006; Cong et al., 2012), was strongly expressed in the nucleus of neurons (Figure 6 A, C, E).

Immunoreactivity of NCL and (NPM3) was reduced in the mediodorsal thalamus in FFI cases when compared with controls (Figure 6 B, D). H3K9me2 expression was also decreased in the thalamus in FFI (Figure 6 F). NCL, NPM3 and H3K9me2 reduction in the mediodorsal thalamus was clearly due to the loss of neurons; reactive glial cells (already represented in previous figures) did not show increased immunoreactivity. Quantitative data are shown in Table IV.

3c. Expression of nucleolin, and initiation and elongation factors, in mediodorsal thalamus

Expression levels of nucleolin were markedly decreased in the mediodorsal thalamus in FFI (Figure 7) as expected from the immunohistochemical studies. Regarding initiation and elongation factors, only eEF1A was significantly decreased in the mediodorsal thalamus of FFI cases (Figure 7). Detailed data are shown in Supplementary Table IV.

Discussion

All the cases examined in the present series are males, have the D178N mutation and are Met/Met homozygous at the codon 129 of *PRNP*. In spite of the variable age at onset, disease duration was between 6 and 9 months; the neuropathological findings were similar from one case to another; therefore, this series conforms a relative homogeneous population. General neuropathological findings in these series are similar to those already described in FFI (14, 21, 25, 27, 29). Based on these features and on the small number of cases, no attempt was made to divide the sample into subgroups.

The post-mortem delay between death and processing was longer in the FFI group when compared with the controls. However, the RIN values were similar in both groups and protein preservation as revealed by western blotting and immunohistochemistry was similar in controls and diseased brains.

Brain hypo-metabolism, as revealed by [18F]-2-fluoro-2-deoxy-D-glucose and positron emission tomography PET to study regional cerebral glucose utilization, predominates in the thalamus and cingulate cortex, but the basal and lateral frontal cortex, the caudate nucleus, and the middle and inferior temporal cortex are also moderately affected (7). The neurodegenerative process starts between 13 and 21 months before the clinical symptoms and it is manifested by hypo-metabolism in the thalamus and impaired thalamic sleep spindle formation in pre-clinically affected family members (6). The present findings are in line with functional studies showing down-regulation of *ATP5D* mRNA expression as revealed by qRT-PCR, and significant reduction in the expression of NDUFB8 (complex I subunit), SDHB (complex II subunit), UQCRC2 (complex III subunit), COX2 (complex IV subunit) and ATP5O (complex V subunit) protein levels, as assessed with western blotting of total homogenates of the mediodorsal thalamus. Immunohistochemistry discloses the origin of reduced mitochondrial expression as being linked to severe neuron loss, as practically no neurons were stained with antibodies against VDAC and ATP5H. These changes are accompanied by increased oxidative stress responses manifested by increased SOD2 immunostaining in reactive astrocytes.

The expression of molecules involved in the machinery of protein synthesis was also altered in the mediodorsal thalamus in FFI when compared with controls. Curiously, increased mRNA expression of *NPM1*, 28S rRNA, *RPS17* and *RPS20* is in contrast to the decreased protein expression levels of the histone-binding chaperones nucleolin (NCL) and nucleoplasmin 3 (NPM3), which regulate rRNA transcription (2, 4, 10, 11, 16, 18, 28), and of histone H3 di-methylated K9 (H3K9me2), which modulates NCL and

NPMs function in rRNA transcription (4, 36). Changes in protein expression are clearly related to the dramatic reduction of neurons as disclosed by immunohistochemistry.

Regarding the protein expression of initiation and elongation factors of protein transcription, only eEF1A is down-regulated. However, the role of this factor is crucial, as elongation occurs when eEF1A is activated following GTP binding and forms a complex with aminoacyl-tRNA, which recognizes the specific sequence in mRNA at the ribosome. Once the interaction of the codon in mRNA with the anti-codon in tRNA is decoded, eEF1A-GDP is hydrolyzed, released from the ribosome, and recycled into its active form by eEF1B. eEF2 assists in the precise codon location at the ribosome (1, 8, 33, 39).

Present molecular studies show reduced expression of proteins linked to the mitochondrial respiratory chain involving subunits encoded by genomic DNA of the five mitochondrial complexes in line with a marked reduction in the number of neurons as revealed by morphological and immunohistochemical studies, as well as decreased metabolism as shown by PET studies in vivo in the mediodorsal nucleus of the thalamus in FFI. Altered machinery of protein synthesis is also manifested by decreased expression of nucleolar chaperones, histone modifications and decreased methylation of DNA in the mediodorsal thalamus in FFI. Alterations in the levels of proteins do not correlate with modifications in the expression of corresponding mRNA levels, which are often maintained or even up-regulated. This may be explained by failed compensatory mechanisms in the remaining neurons or by increased RNA expression in reactive astrocytes. In favor of the latter, is the dramatic increase of reactive astrocytes and the increased expression of superoxide dismutase 2 which tags reactive astrocytes as crucial components of oxidative stress responses in the mediodorsal thalamus in FFI.

All these changes are observed at terminal stages of the disease, with devastating effects on selected brain regions. Little can be done at this stage but efforts must be made at preclinical stages to prevent mitochondrial and protein synthesis havoc at advanced stages of the disease.

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Table I: Summary of the main clinical and pathologic features of cases used in the present study. PMD: post-mortem delay; C: control; FFI; fatal familial insomnia; RIN: RNA integrity number; Thal: mediodorsal thalamus

Case	Sex	Age	PMD	Neuropathology	RIN Thal
1	M	67	14h40min	C	6
2	M	61	4h30min	C	7.2
3	M	59	4h15min	C	6.6
4	W	66	12h10min	C	6.7
5	M	76	4h15min	C	6
6	M	81	11h40min	C	6
7	M	57	5h	C	6.9
8	M	53	12h10min	FFI	7.9
9	M	56	22h50min	FFI	5.3
10	M	36	17h30min	FFI	6.4
11	M	70	15h30min	FFI	-
12	M	50	12h30min	FFI	6.8
13	M	47	3h30min	FFI	8.2
14	M	54	9h50min	FFI	7.5

Table II: Abbreviated names of genes, their full names, and TaqMan probe references used for the study of mRNA expression of mitochondria, protein synthesis and purine metabolism enzymes including GUS- β and XPNPEP1 used for normalisation.

Gene	Gene full name	Taqman probes
<i>XPNPEP1</i>	X-propylaminopeptidase 1	Hs00958026_m1
Mitochondria		
<i>NDUFA2</i>	NADH Dehydrogenase (Ubiquinone) 1 apha subcomplex, 2, 8kDa	Hs04187282_g1
<i>NDUFA7</i>	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 7, 14.5kDa	Hs01561430_m1
<i>NDUFA10</i>	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10, 42kDa	Hs01071117_m1
<i>NDUFB3</i>	NADH Dehydrogenase (Ubiquinone) 1 Beta subcomplex, 3, 12kDa	Hs00991297_g1
<i>NDUFB7</i>	NADH Dehydrogenase (Ubiquinone) 1 Beta subcomplex, 7, 18kDa	Hs00188142_m1
<i>NDUFB10</i>	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10, 22kDa	Hs00605903_m1
<i>NDUFS7</i>	NADH dehydrogenase (ubiquinone) Fe-S protein 7, 20kDa (NADH-coenzyme Q reductase)	Hs00257018_m1
<i>NDUFS8</i>	NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23kDa (NADH-coenzyme Q reductase)	Hs00159597_m1
<i>SDHB</i>	Succinate dehydrogenase complex, subunit B, Iron Sulfur (lp)	Hs01042482_m1
<i>UQCRI1</i>	Ubiquinol-cytochrome C reductase, complex III subunit XI	Hs00199138_m1
<i>UQCRB</i>	Ubiquinol-cytochrome C reductase binding protein	Hs01890823_s1
<i>COX7A2L</i>	cytochrome c oxidase subunit VIIa polypeptide 2 like	Hs00190880_m1
<i>COX7C</i>	Cytochrome C oxidase subunit VIIc	Hs01595220_g1
<i>ATP5D</i>	ATP Synthase, H ⁺ transporting, mitochondrial F1 complex, Delta Subunit	Hs00961521_m1
<i>ATP5G2</i>	ATP synthase, H ⁺ transporting, mitochondrial Fo complex, subunit C2	Hs01086654_g1
<i>ATP5H</i>	ATP Synthase, H ⁺ transporting, mitochondrial Fo complex, subunit D	Hs01046892_gH
<i>ATP5L</i>	ATP Synthase, H ⁺ transporting, mitochondrial Fo complex, subunit G	Hs00538946_g1
<i>ATP5O</i>	ATP Synthase, H ⁺ transporting, mitochondrial F1 complex, O subunit	Hs00426889_m1
<i>ATP6V0B</i>	ATPase, H ⁺ transporting, lysosomal 21kDa, V0 subunit b	Hs01072388_m1
<i>ATP6V1H</i>	ATPase, H ⁺ transporting, lysosomal 50/57kDa, V1 subunit H	Hs00977530_m1
<i>TOMM40</i>	Translocase of outer mitochondrial membrane 40	Hs01587378_mH
Protein synthesis		
<i>NCL</i>	nucleolin	Hs01066668_m1

<i>NPM1</i>	nucleophosmin /protein B23)	Hs02339479_g1
<i>UBTF</i>	eukaryotic upstream binding factor	Hs01115792_g1
<i>18S rRNA</i>	eukaryotic 18S rRNA	Hs99999991_s1
<i>28S rRNA</i>	RNA, 28S ribosomal	Hs03654441_s1
<i>RPL5</i>	ribosomal protein L5	Hs03044958_g1
<i>RPL7</i>	ribosomal protein L7	Hs02596927_g1
<i>RPL21</i>	ribosomal protein L21	Hs00823333_s1
<i>RPL22</i>	ribosomal protein L22	Hs01865331_s1
<i>RPL23A</i>	ribosomal protein L23A	Hs01921329_g1
<i>RPL26</i>	ribosomal protein L26	Hs00864008_m1
<i>RPL27</i>	ribosomal protein L27	Hs03044961_g1
<i>RPL30</i>	ribosomal protein L30	Hs00265497_m1
<i>RPL31</i>	ribosomal protein L31	Hs01015497_g1
<i>RPS3A</i>	ribosomal protein S3A	Hs00832893_sH
<i>RPS5</i>	ribosomal protein S5	Hs00734849_g1
<i>RPS6</i>	ribosomal protein S6	Hs04195024_g1
<i>RPS10</i>	ribosomal protein S10	Hs01652370_gH
<i>RPS13</i>	ribosomal protein S13	Hs01011487_g1
<i>RPS16</i>	Ribosomal protein S16	Hs01598516_g1
<i>RPS17</i>	ribosomal protein S17	Hs00734303_g1
<i>RPS20</i>	Ribosomal protein S20	Hs00828752_gH

Table III: List of antibodies used for western blotting and immunohistochemistry.

Antibody	Reference	Supplier	Host	wb dilution	ihq dilution
Nucleolin	Ab22758	Abcam (Cambridge, UK)	rb	1/100	1/1,000
anti-eukaryotic translation initiation factor 2 (eIF2- α)	5A5	Thermo Scientific (Waltham, MA, USA)	m	1/50	
anti-phospho-eIF2-alpha pSer51 (p-EIF2- α)	S.674.5	Thermo Scientific (Waltham, MA, USA)	rb	1/50	
anti-eukaryotic translation initiation factor 5 (eIF5)	sc-282	Santa Cruz (Dallas, Texas, USA)	rb	1/400	
anti-eukaryotic elongation factor 1A (eEF1A)	2551	Cell signaling (Danvers, MA, USA)	rb	1/100	
anti-eukaryotic elongation factor 2 (eEF2)	2332	Cell signaling (Danvers, MA, USA)	rb	1/500	
Total OXPHOS Antibody Cocktail	ab110411	Mitosciences, Abcam (Cambridge, UK)	m	1/1,000	
ATP5O (OSCP Antibody)	10994-1-AP	Proteintech (Chicago, USA)	rb	1/1,000	
UQCRB	10756-1-AP	Proteintech (Chicago, USA)	rb	1/250	
VDAC1	Ab15895	Abcam (Cambridge, UK)	rb	1/400	1/100
β -actin	A5316	Sigma-Aldrich (St Louis, MO, USA)	m	1/5,000	
ATP5H	17589-1-AP	Proteintech (Chicago, USA)	rb		1/100
Anti-Manganese superoxide dismutase SOD2	SOD-110	Stressgen (San Diego, USA)	rb		1/100
Anti-Histone H3K9me2	Ab1220	Abcam (Cambridge, UK)	m		1/90

Table IV: Quantitative data of cells stained with Nissl stain and immunoreactive with antibodies against voltage dependent anion channel (VDAC), glial fibrillary acidic protein (GFAP), CD8 (reactive microglia), nucleolin (NCL), nucleoplasmin 3 (NPM3), histone H3K9me2, ATP5H protein and superoxide dismutase 2 (SOD2) in the mediodorsal thalamus in control and FFI cases. Values are expressed as mean values \pm SD. Assessed cell types are named in the left column excepting ATP5H and SOD2 immunoreactive cells which are neurons in controls and glia in FFI cases.

	Control	FFI
Nissl (only neurons)	20.5 \pm 2.01	0.9 \pm 0.73
VDAC (only neurons)	20.4 \pm 2.06	1.3 \pm 0.94
GFAP (astrocytes)	8.8 \pm 2.09	35 \pm 6.87
CD68 (reactive microglia)	2.3 \pm 0.94	18.4 \pm 3.40
NCL (only neurons)	6.8 \pm 1.81	0
NPM3 (only neurons)	6 \pm 1.82	0
H3K9me2 (only neurons)	21.8 \pm 2.04	0
ATP5H	23.7 \pm 2.45 (neurons)	23 \pm 3.52 (glia)
SOD2	20.6 \pm 1.83 (neurons)	25.5 \pm 2.83 (glia)

Figure legends

Figure 1: Mediodorsal thalamus A, B, E, F, I, J, M, N) and entorhinal cortex C, D, G, H, K, L, O, P) in age-matched control (A, C, E, G, I, K, M, O) and FFI (B, D, F, H, J, L, N, P) cases. Severe neuron loss is seen in the mediodorsal thalamus in FFI, whereas moderate neuron loss and spongiform change is observed in the entorhinal cortex in FFI. Increased numbers of GFAP-immunoreactive astrocytes are present in mediodorsal thalamus and entorhinal cortex in FFI compared with corresponding controls. Increased numbers of Iba1-immunoreactive cells and CD-68-positive cells are seen in the EC in six cases and in two of seven FFI cases (here illustrated) compared with controls. Paraffin sections, A-D: haematoxylin and eosin; E-H: GFAP immunohistochemistry; I-L: Iba1 immunohistochemistry; M-P: CD-68 immunohistochemistry; immunohistochemical sections slightly counterstained with haematoxylin. A-P, bar in P = 55 μ m. D1, H1, L1, P1, bar in P1 = 30 μ m.

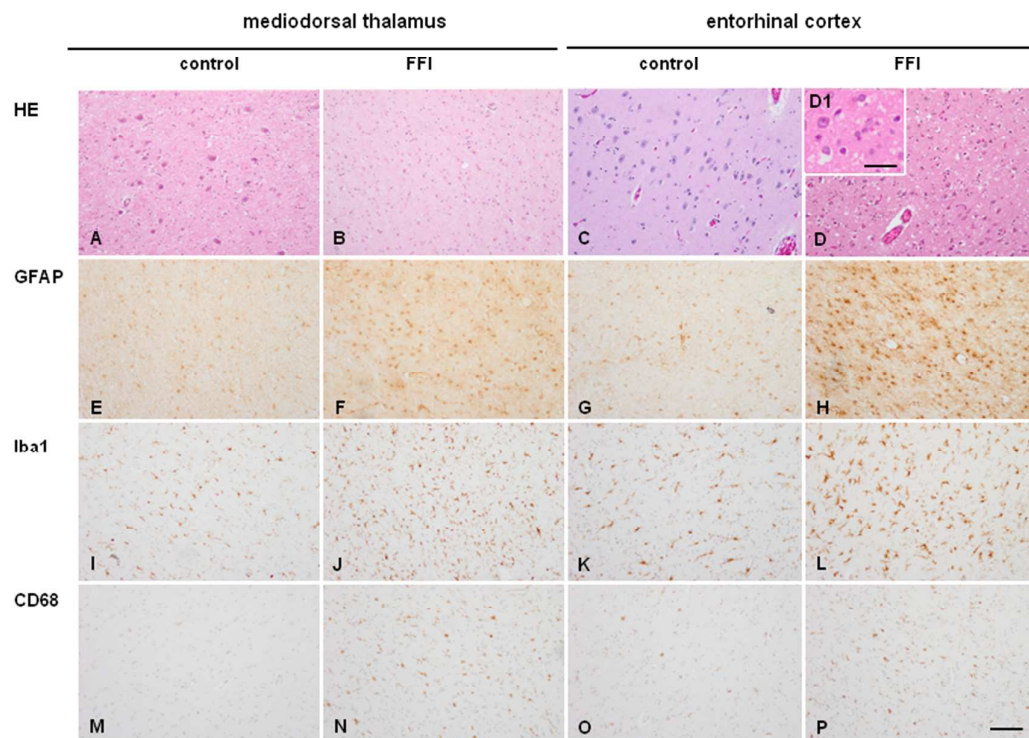


Figure 2: Mediodorsal nucleus of the thalamus (thal: A-C) and entorhinal cortex (EC: D-F) in control (A, B) and FFI (B, C, E, F) cases showing marked loss of neurons without spongiform change in the thalamus and moderate neuron loss and spongiform change in entorhinal cortex in FFI when compared with corresponding regions in controls. In FFI, no proteinase K-resistant PrP (PrPRes) is observed in the mediodorsal thalamus (C) whereas punctate, synaptic-like immunostaining is observed in the entorhinal cortex (F) processed in parallel. Paraffin sections, A, B, D, E Nissl staining, bar in E = 50 μ m; C, F, PrP immunohistochemistry (3F4 antibody) following pre-incubation with proteinase K, bar in F = 25 μ m.

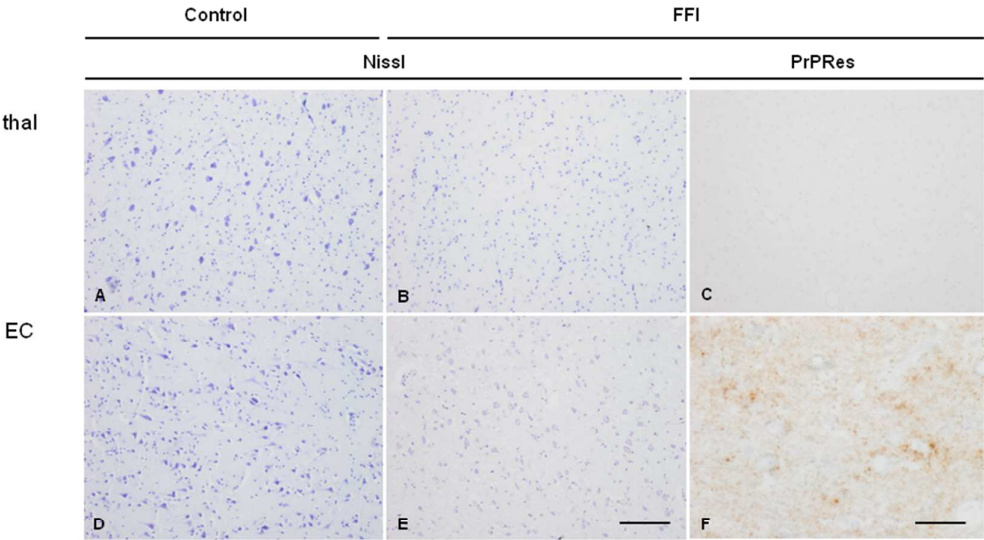
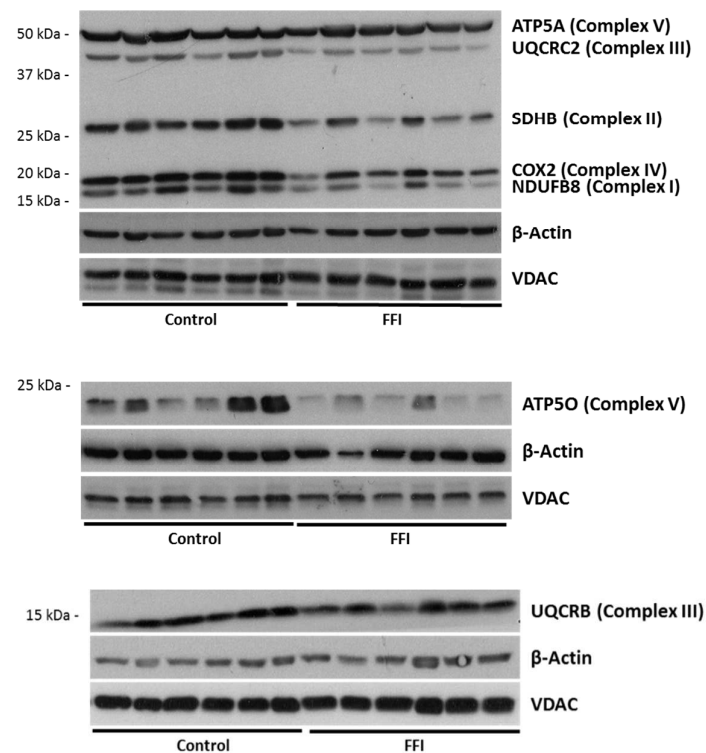


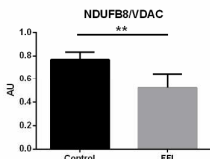
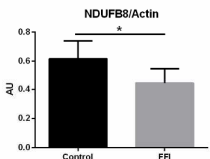
Figure 3: A) Western blot of subunits NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8, 19 kDa (NDUFB8), succinate dehydrogenase complex, subunit B, iron sulphur (lp) (SDHB), ubiquinol-cytochrome c reductase core protein II (UQCRC2), cytochrome c oxidase subunit II (COX2) and ATP synthase, H⁺ transporting, mitochondrial F1 complex, and alpha subunit 1 (ATP5A) using OXPHOS antibody; ATP synthase, H⁺ transporting mitochondrial F1 complex, O subunit (ATP5O) and ubiquinol-cytochrome c reductase binding protein (UQCRB) antibodies in mediodorsal thalamus in FFI and control cases. β -actin and VDAC were used to normalize total protein and mitochondria protein loading, respectively.



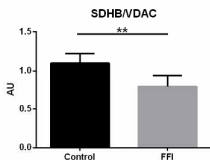
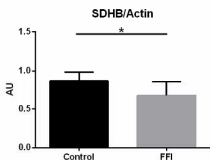
B) Densitometric analysis shows significant reduction of NDUFB8, SDHB, UQCRC2 and COX2 normalized with β -actin and VDAC in FFI compared with controls. ATP5A and UQCRB protein levels were preserved. Results were analysed by Graphpad Prism with Student's t test when the distribution was normal and with Mann-Whitney test if distribution was not normal as assessed with the Kolmogorov-Smirnov normality test. Differences are considered statistically significant at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. ATP5O shows a significant reduction when normalized with β -actin but only a trend with VDAC.

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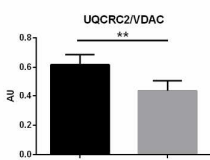
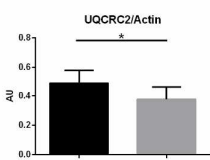
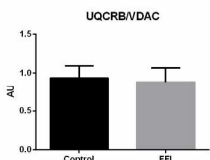
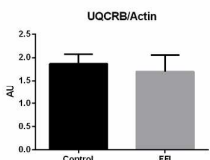
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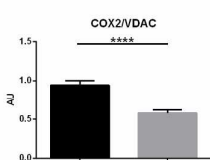
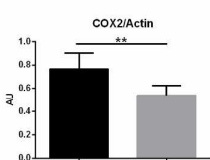
Complex II



Complex III



Complex IV



Complex V

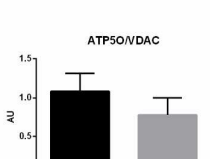
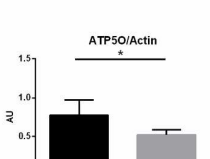
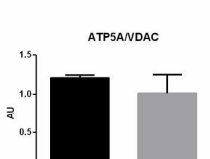
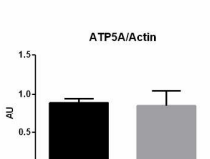


Figure 4: Mediodorsal nucleus of the thalamus in control (A, C, E, F) and FFI (B, D, F, H) cases. Nissl staining (A, B) shows marked decrease in the number of neurons in FFI when compared with controls. (VDAC) (C, D), (C, D) (SOD2) (E, F) immunoreactivity is found in mediodorsal thalamus in control (A, C, E) and FFI (B, D, F) cases). Decreased voltage-dependent anion channel (VDAC) (C, D) and ATP synthase, H⁺ transporting, mitochondrial Fo complex, subunit d (ATP5H) (E, F) immunoreactivity is found in the mediodorsal thalamus in FFI compared to controls due to the dramatic decrease in the number of neurons. However, ATP5H is observed in reactive glial cells. Weak superoxide dismutase 2 (SOD2) (G, H) immunoreactivity is seen in neurons in control cases which contrasts with enhanced SOD2 immunostaining in reactive astrocytes in FFI. Paraffin sections, A, B: Nissl staining; C-H immunohistochemical sections slightly counterstained with haematoxylin. A-H, bar in H = 25 μ m. F1, H1, bar in H1 = 10 μ m.

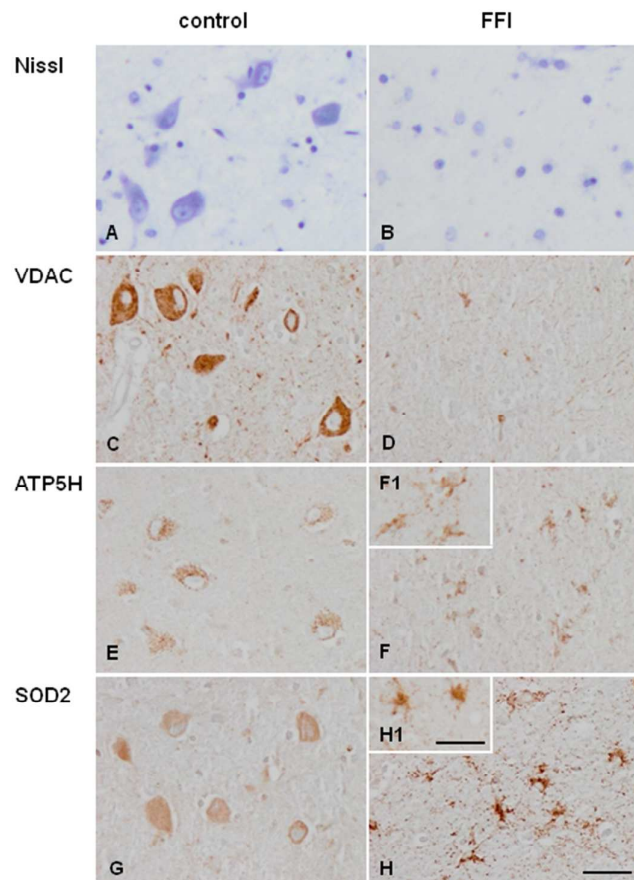


Figure 5: mRNA expression of ribosomal proteins (RPL and RPS) in mediodorsal thalamus in FFI and controls. *RPS17*, *RPS20*, *NPM1* and *28S rRNA* are up-regulated in FFI cases using *XPNPEP1* for normalisation. Mean fold-change values for each group are compared with Student's t test and differences were considered statistically significant at * $p < 0.05$.

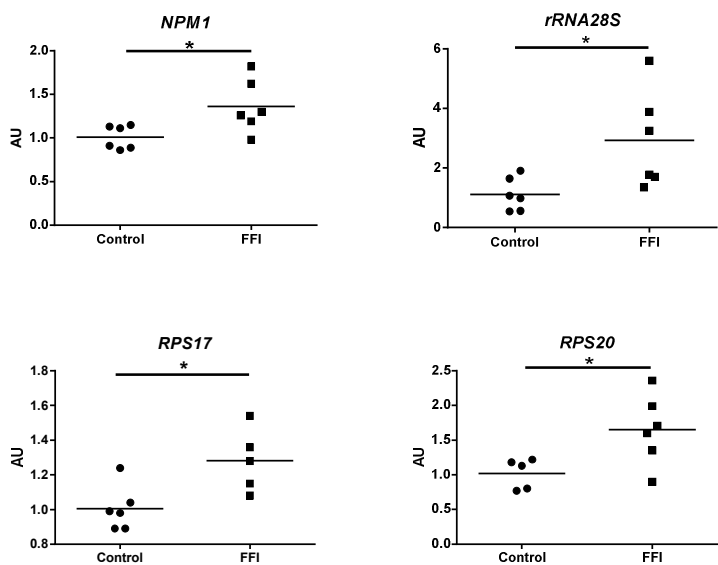


Figure 6: Nucleolin (NCL) (A, B), NPM3 (C, D) and H3 dimethyl K9 (H3K9me2) (E, F) immunoreactivity is observed in neurons in the mediodorsal thalamus in controls (A, C, E). Weak NCL immunoreactivity is found in the nucleolus whereas strong NCL immunoreactivity is present in the neuronal cytoplasm. NPM3 immunoreactivity is observed in the nucleolus, whereas H3K9me2 immunolabelling is localized in the nucleus. Decreased NCL, NPM3 and H3K9me2 expression is found in FFI cases (B, D, E) due to the marked decrease in the number of neurons. Paraffin sections slightly counterstained with haematoxylin; bar = 25 μ m.

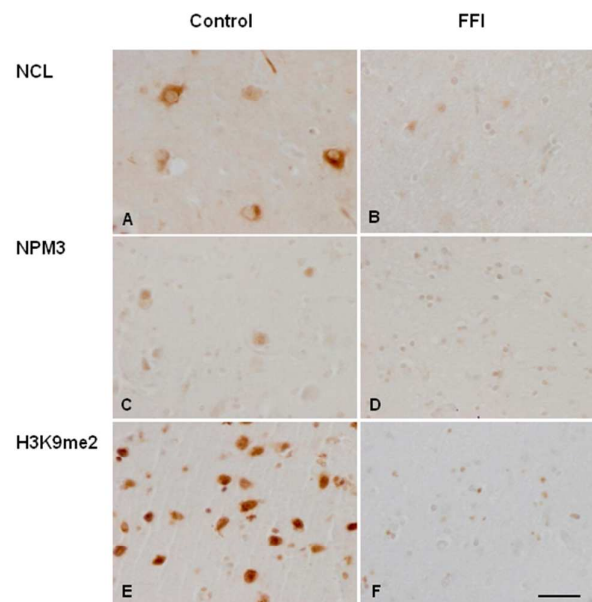
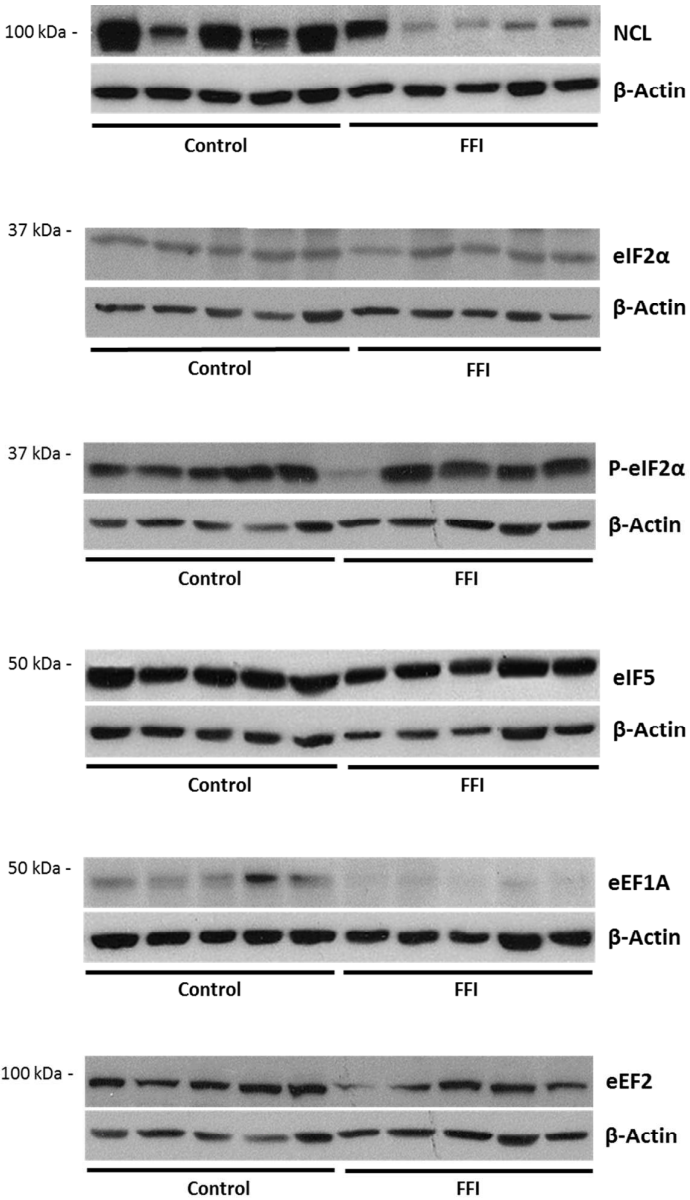
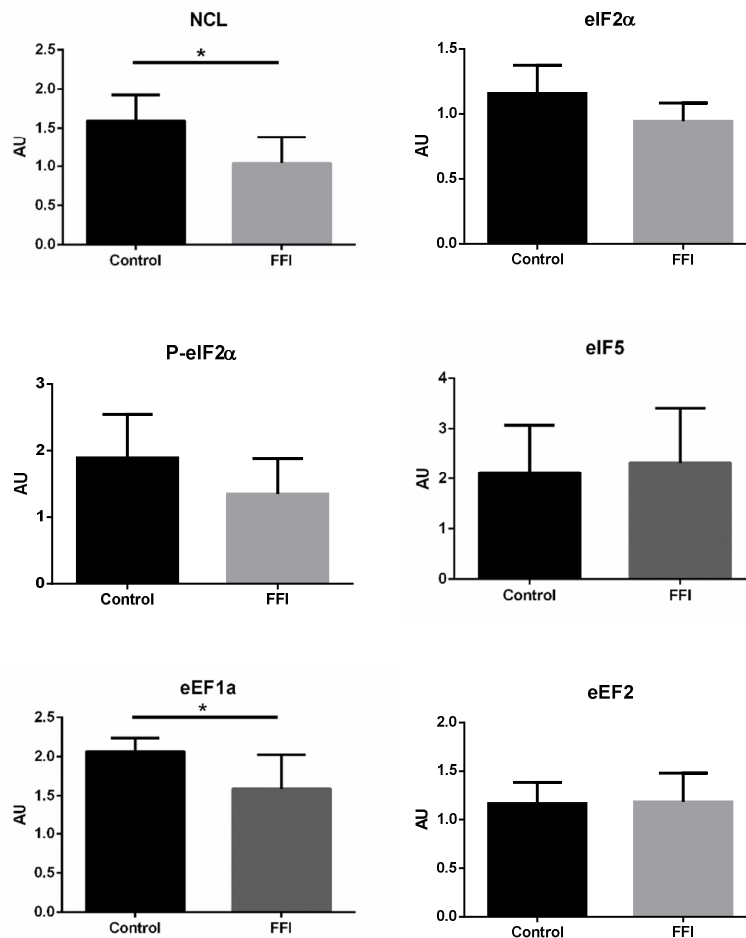


Figure 7: A) Western blots of nucleolin, initiation (eIF2 α , P- eIF2 α , eIF5) and elongation factors (eEF1A, eEF2) in mediodorsal thalamus in FFI and control cases. β -actin was used to normalize total protein.



B) Densitometric analysis shows significant reduction of NCL and eEF1A. Results were analysed by Graphpad Prism with Student's t test when the distribution was normal and with Mann-Whitney test if distribution was not normal as assessed with the Kolmogorov-Smirnov normality test. Differences are considered statistically significant at * $p < 0.05$.



Supplementary Tables

Supplementary Table I: Gene expression of subunits of mitochondrial complexes in the mediodorsal thalamus in FFI and control cases normalized with XPNPEP1.

Thalamus	Probe	Control	FFI	P value	
Complex I	<i>NDUFA2</i>	1.02 ± 0.20	0.79 ± 0.17	0.06	-
	<i>NDUFA7</i>	1.02 ± 0.20	0.91 ± 0.34	0.50	-
	<i>NDUFA10</i>	1.00 ± 0.10	0.96 ± 0.13	0.49	-
	<i>NDUFB3</i>	1.02 ± 0.22	0.82 ± 0.15	0.10	-
	<i>NDUFB7</i>	1.02 ± 0.2	0.87 ± 0.26	0.31	-
	<i>NDUFB10</i>	1.02 ± 0.21	0.89 ± 0.23	0.34	-
	<i>NDUFS7</i>	1.02 ± 0.22	0.91 ± 0.20	0.37	-
	<i>NDUFS8</i>	1.01 ± 0.19	0.95 ± 0.41	0.73	-
Complex II	<i>SDHB</i>	1.03 ± 0.24	0.87 ± 0.17	0.21	-
Complex III	<i>UQCR11</i>	1.03 ± 0.26	0.94 ± 0.19	0.50	-
	<i>UQCRB</i>	1.06 ± 0.38	0.78 ± 0.31	0.20	-
Complex IV	<i>COX7A2L</i>	1.03 ± 0.27	0.87 ± 0.15	0.25	-
	<i>COX7C</i>	1.05 ± 0.37	1.01 ± 0.20	0.81	-
Complex V	<i>ATP5D</i>	1.02 ± 0.22	0.63 ± 0.08	0.004	**
	<i>ATP5G2</i>	1.01 ± 0.13	1.36 ± 0.48	0.09	-
	<i>ATP5H</i>	1.02 ± 0.21	0.85 ± 0.20	0.18	-
	<i>ATP5L</i>	1.01 ± 0.19	0.98 ± 0.20	0.76	-
	<i>ATP5O</i>	1.02 ± 0.19	0.80 ± 0.19	0.09	-
	<i>ATP6V0B</i>	1.06 ± 0.39	0.87 ± 0.15	0.34	-
	<i>ATP6V1H1</i>	1.13 ± 0.64	0.73 ± 0.13	0.21	-
	<i>TOMM40</i>	1.03 ± 0.25	0.80 ± 0.12	0.08	-

Supplementary Table II: Densitometric values of expression of mitochondrial proteins as revealed by western blotting in the mediodorsal thalamus in FFI cases and controls normalized with β -actin (A) and VDAC (B).

A

Protein	Control	FFI	P value	
NDUFB8	0.62 \pm 0.12	0.44 \pm 0.11	0.0257	*
SDHB	0.87 \pm 0.11	0.65 \pm 0.18	0.0426	*
UQCRB	1.87 \pm 0.21	1.70 \pm 0.36	0.34	-
UQCRC2	0.49 \pm 0.09	0.38 \pm 0.09	0.0462	*
COX2	0.77 \pm 0.14	0.54 \pm 0.09	0.0071	**
ATP5A	0.89 \pm 0.05	0.85 \pm 0.19	0.69	-
ATP5O	0.78 \pm 0.20	0.52 \pm 0.07	0.0227	*

B

Protein	Control	FFI	P value	
NDUFB8	0.77 \pm 0.06	0.52 \pm 0.12	0.0087	**
SDHB	1.10 \pm 0.13	0.79 \pm 0.15	0.0035	**
UQCRB	0.94 \pm 0.16	0.88 \pm 0.18	0.61	-
UQCRC2	0.62 \pm 0.07	0.44 \pm 0.07	0.0022	**
COX2	0.94 \pm 0.06	0.58 \pm 0.04	0.0001	****
ATP5A	1.21 \pm 0.04	1.02 \pm 0.23	0.08	-
ATP5O	1.08 \pm 0.23	0.78 \pm 0.22	0.09	-

Supplementary Table III: Gene expression of nucleolar proteins. 18S and 28S rRNAs and ribosomal proteins in the mediodorsal thalamus in FFI and control cases normalized with XPNPEP1.

Genes encoding for	Probe	Control	FFI	P Value	
Nucleolar proteins	<i>UBTF</i>	1.00 ± 0.05	0.88 ± 0.12	0.09	-
	<i>NCL</i>	1.01 ± 0.17	1.05 ± 0.12	0.69	-
	<i>NPM1</i>	1.01 ± 0.14	1.36 ± 0.31	0.027	*
rRNAs	<i>rRNA18S</i>	1.01 ± 0.19	1.30 ± 0.46	0.24	-
	<i>rRNA28S</i>	1.12 ± 0.56	2.93 ± 1.64	0.0287	*
Ribosomal proteins	<i>RPL5</i>	1.01 ± 0.14	1.17 ± 0.15	0.09	-
	<i>RPL7</i>	1.03 ± 0.25	1.27 ± 0.11	0.13	-
	<i>RPL21</i>	1.05 ± 0.34	0.87 ± 0.37	0.40	-
	<i>RPL22</i>	1.00 ± 0.09	1.48 ± 0.56	0.09	-
	<i>RPL23A</i>	1.03 ± 0.27	1.14 ± 0.10	0.42	-
	<i>RPL26</i>	1.03 ± 0.27	0.80 ± 0.44	0.13	-
	<i>RPL27</i>	1.03 ± 0.25	1.44 ± 0.46	0.09	-
	<i>RPL30</i>	1.03 ± 0.27	1.33 ± 0.22	0.08	-
	<i>RPL31</i>	1.03 ± 0.30	1.23 ± 0.15	0.21	-
	<i>RPS3A</i>	1.02 ± 0.22	1.20 ± 0.21	0.19	-
	<i>RPS5</i>	1.01 ± 0.19	1.55 ± 0.63	0.07	-
	<i>RPS6</i>	1.00 ± 0.04	1.53 ± 0.59	0.08	-
	<i>RPS10</i>	1.01 ± 0.12	1.04 ± 0.15	0.66	-
	<i>RPS13</i>	1.02 ± 0.22	1.17 ± 0.16	0.24	-
	<i>RPS17</i>	1.01 ± 0.13	1.28 ± 0.18	0.016	*
	<i>RPS20</i>	1.02 ± 0.22	1.65 ± 0.51	0.0295	*

Supplementary Table IV: Densitometric values of expression of initiation and elongation factors of protein transcription in the mediodorsal thalamus in FFI cases and controls normalized with β -actin

Protein	Control	FFI	P value	
NCL	1.60 \pm 0.33	1.04 \pm 0.34	0.012	*
eIF2 α	1.16 \pm 0.22	0.95 \pm 0.14	0.06	-
P- eIF2 α	1.90 \pm 0.65	1.35 \pm 0.53	0.13	-
eIF5	2.12 \pm 0.95	2.33 \pm 1.08	0.72	-
eEF1A	2.07 \pm 0.17	1.59 \pm 0.44	0.021	*
eEF2	1.17 \pm 0.22	1.18 \pm 0.30	0.91	-